Repurposing libraries of eukaryotic protein kinase inhibitors for antibiotic discovery

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With the rise in resistance that inevitably follows the clinical deployment of an antibiotic, there is a continual need for new antibiotic discovery, development and approval. Food and Drug Administration approvals for Synercid (quinupristin/dalfopristin) in 1999, Zyvox (linezolid) in 2000, and Cubicin (daptomycin) in 2003 have addressed life-threatening infections from drug-resistant Gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus pneumomiae*, and *Enterococcus faecalis*. However, because these antibiotics are not active against an emerging class of nosocomial pathogens (multidrug-resistant Gram-negative bacteria, including strains of *Klebsiella*, *Acinetobacter* and *Pseudomonas*) there is renewed focus on developing treatments for infections caused by Gram-negative bacteria. To this end, in this issue of PNAS Miller *et al.* (1) report a novel approach to discovering new classes of antibiotics.

Although bacterial genome sequencing and genetics have identified essential genes as potential targets for new antibiotics, efforts to screen synthetic chemical libraries have been disappointingly unproductive (2). One possible explanation for the low yield is the bias, historical and contemporary, of pharmaceutical companies' synthetic small molecule libraries for eukaryotic rather than prokaryotic targets. Miller *et al.* (1) sought to turn this paradigm on its head by making a virtue of the depth of such a library, in this case composed of protein kinase inhibitors. Even though protein kinases are much less widespread in bacterial metabolism, Miller *et al.* set out to determine whether their library contained ATP analogs that could kill bacterial cells potently and selectively.

The Pfizer team (1) made use of the company's screening file, comprising some 1.6 million compounds. A significant number of these compounds were heterocycles designed to be ATP-competitive ligands for the ATP binding site of a eukaryotic protein kinase. They screened their library against a membrane-compromised (*imp*), efflux pump-deficient (*tolC*) strain of *Escherichia coli* to find molecules that entered and killed whole cells. Their choice of indicator strain imparted a focus on molecules with anti-Gram-negative activity from the outset.

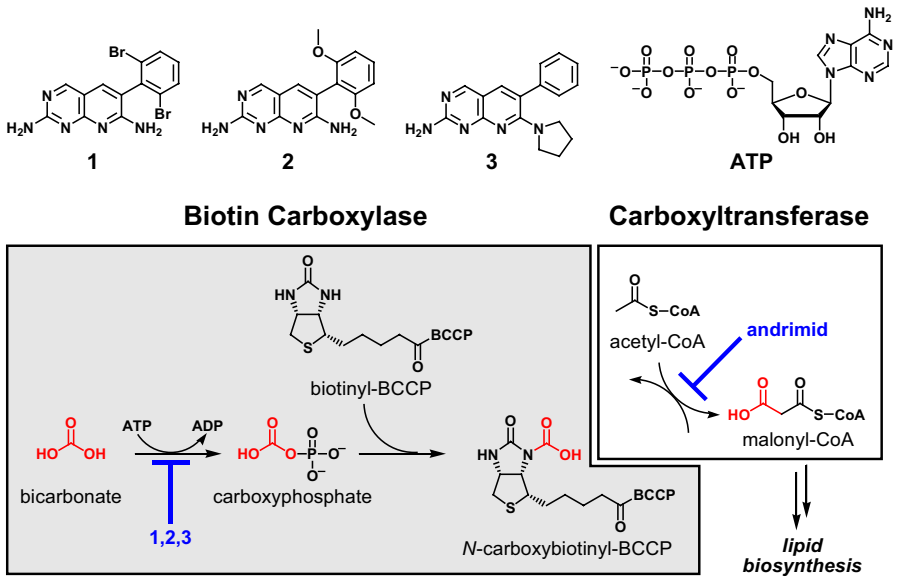

Fig. 1. Small molecule inhibitors of acetyl-CoA carboxylase. (*Upper*) The chemical structures of molecules 1–3 and ATP are shown. (*Lower*) A schematic of the reaction catalyzed by acetyl-CoA carboxylase, divided into the portions catalyzed by its biotin carboxylase and carboxyltransferase subunits.

Miller *et al.* (1) identified promising hits from a series of pyridopyrimidines and determined that drug-resistant mutants (1 in 10^9) mapped to the biotin carboxylase (BC) subunit of the multisubunit enzyme acetyl-CoA carboxylase, which catalyzes the first committed step in fatty acid biosynthesis (Fig. 1). Intriguingly, the pseudopeptide natural product andrimid is a nanomolar inhibitor of the carboxyltransferase subunit of the same enzyme (3), raising the prospect of the synergistic action of 2 drugs against this essential enzyme.

Bacterial fatty acid biosynthesis has long been thought to be an underexploited pathway for antibiotics. Although 2 inhibitors of the enoylreductase component of fatty acid synthase (FAS) (isoniazid, used in combination therapies for tuberculosis, and triclosan, an antiseptic) have seen use in humans, no other clinically-used antibiotics target this pathway. However, recent efforts to identify inhibitors of fatty acid biosynthesis have yielded promising leads: a screen of natural product extracts at Merck turned up the *Streptomyces* metabolite platensimycin as a potent inhibitor of the FAS chain elongation enzyme (4).

The BC subunit of acetyl-CoA carboxylase carries out this enzyme's first half-reaction: activating bicarbonate by cleaving ATP to ADP and transiently generating the mixed anhydride carboxyphosphate. This activated carboxyl moiety is then captured by biotin [which is tethered to the biotin carboxyl carrier protein (BCCP)], to yield *N*-carboxybiotinyl-BCCP (Fig. 1). A second subunit, the carboxyltransferase, generates the thioester enolate of acetyl-CoA and catalyzes its attack on the biotin-tethered carboxyl group to generate malonyl-CoA, the building block for fatty acid synthesis. The BC subunit, which had been crystallized (5), is a member of the ATP-grasp enzyme superfamily. The crystal structures of library compounds **1** and **2** in complex with the BC subunit confirmed that they bind to its ATP-binding site.

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See companion article on page 1737.

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Hit molecules from the screen were bactericidal against *E. coli* and the consequential Gram-negative respiratory pathogens *Haemophilus influenzae* and *Moraxella catarrhalis*, but not against Gram-positive bacteria. Compound **1** has a K_d of 0.8 nM against the purified BC subunit from *E. coli*, and the selectivity profile of compounds **1** and **2** is encouraging: no significant inhibition of 30 eukaryotic protein kinases, including VEGFR-2 and FGFR-1, the targets for which these heterocyclic scaffolds had been optimized. Thus, the ATP-binding sites of eukaryotic and prokaryotic enzymes may be an expanding class of valuable targets for molecules with nanomolar potency and selectivity.

One of the ATP-binding site mutations in BC that conferred pyridopyrimidine resistance was I437T. BC subunits from Gram-positive bacteria harbor a threonine at this position, providing a possible explanation for why compounds **1** and **2** are selective for Gram-negative bacteria. Further examination of the library, leveraging the prior medicinal chemical investment by Pfizer in eukaryotic tyrosine kinase inhibitors, showed that compound **3**, with a K_d of 150 nM, was only 14-fold less potent against the I437T BC mutant. This result gives a clear path for future medicinal chemical efforts to tailor the pyridopyrimidine scaffold to get higher efficacy against BCs from Gram-positive pathogens such as *S. aureus*.

Initial pharmacokinetic studies in mice led to the demonstration of efficacy in both a thigh model and a systemic model of murine infection with *H. influenzae*. Anticipating that a BC inhibitor might be a candidate for combination therapy, compound **1** was tested for

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antagonism or synergy with a several antibiotics; the antiseptic triclosan and the DNA gyrase inhibitor ciprofloxacin were synergistic. Because andrimid inhibits the other catalytic subunit of acetyl-CoA carboxylase (Fig. 1), it might be of interest to evaluate it in combination with the pyridopyrimidines for potential synergy.

In addition to spotlighting BC as a new antibacterial target with subnanmolar lead compounds from a synthetic library, one of the values of Miller *et al.*'s study (1) is the renewed impetus it places on the evaluation of other bacterial enzymes that use ATP (or GTP) as potential targets for new antibiotics. To wit: the target of ciprofloxacin and other fluoroquinolones is the ATP-consuming topoisomerase DNA gyrase, an inhibitor of the *M. tuberculosis* ATP synthase (6) is in late-stage clinical evaluation, and semisynthetic derivatives of the ADEP family of natural products allosterically activate ATP-dependent chambered proteases (7).

Both DNA gyrase and BC are in the ATP-grasp superfamily, members of which cleave ATP to ADP. D-Ala-D-Ala ligase, an ATP-grasp enzyme involved in muropeptide elongation, is targeted with modest affinity by members of a synthetic screening library (8). It may be worthwhile to test all bacterial ATP-grasp enzymes with libraries of synthetic kinase inhibitors. In this vein, the predominant form of protein kinases in bacteria are the autophosphorylating histidine kinases involved in 2-component signaling pathways. Although no promising inhibitor candidates have emerged against this class to date, Miller *et al.*'s results (1) suggest that rescreening with eukaryotic target-directed ATP-mimetic libraries would be worthwhile. It is unclear how many ATP-using enzymes in the bacterial proteome, both phosphoryl transferases and adenylyl transferases, could be targeted by existing eukaryotic kinase libraries. However, it is promising that ATP-mimetic scaffolds have been particularly malleable for exploring target protein selectivity while maintaining high potency.

Finally, the question arises whether a similar strategy of using synthetic chemical libraries developed and iteratively improved for other classes of eukaryotic proteins might be profitably screened against bacterial targets in whole-cell assays. Two target classes that might be worth particular attention are ion channels and prenyltransferases (9).

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